Deep Brain Stimulation of the Nucleus Accumbens for the Treatment of Cocaine Addiction

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Table of contents

List of tables

List of figures

Abbreviations

5HT	serotonin
ANOVA	analysis of variance
AP	anteroposterior
cAMP	cyclic-AMP
DA	dopamine
DAT	dopamine transporter
DBS	deep brain stimulation
DPX	di-butyl phthalate xylene
DV	dorsoventral
FR	fixed-ratio
HF	high frequency stimulation
i.p	intra-peritoneal
LF	low frequency stimulation
ML	mediolateral
NAc	nucleus accumbens
NE	norepinephrine
OCD	obsessive-compulsive disorder
OFC	orbitofrontal cortex
PB	phosphate buffer
PD	Parkinson's disease
PFA	paraformaldehyde
PFC	prefrontal cortex
s.c	sub cutaneous
VTA	ventral tegmental area

Abstract

With approximately 7% of the adult population reporting to have taken illicit substances over the course of a year and the chronically relapsing nature of substance use disorders there is a great need for effective forms of treatment and therapies to reduce relapse. Deep brain stimulation (DBS) is a process of neuromodulation where electrodes are implanted in a target region to modulate the electrophysiological activity of the target region. DBS has been postulated as a potential therapy for treatment-refractory addiction, with a great deal of focus on the nucleus accumbens (NAc). Forty male Long Evans rats were implanted with unilateral stimulating electrodes within the right NAc prior to exposure to chronic cocaine self-administration (0.5 mg/kg/infusion). Following self-administration, the animals were withdrawn from cocaine and treated with 14 consecutive days of sham, low frequency (LF, 20 Hz) or high frequency (HF, 160 Hz) stimulation sessions (30 min/day). The animals underwent drug seeking tests on days 1, 15 and 30 of the withdrawal phase with context-induced relapse paired with a drug challenge (5 mg/kg i.p). Relapse rates were highest on day 15 after withdrawal, with both LF and HF attenuating cocaine during this drug-seeking test, however this was not the case for tests on days 1 and 30. Motivation to respond for saccharin solution (0.1 %) remained intact following both LF and HF stimulation intake sessions. These results demonstrate that unilateral DBS of the NAc effectively attenuated cocaine-seeking following chronic exposure to stimulation although these beneficial effects appeared to diminish following cessation of daily treatment with stimulation. The results obtained in this experiment provide support for DBS as a potential therapy for patients with treatment-resistant cases of substance use disorders.

1.0 Introduction

1.1 General Overview

Drug addiction, referred to as substance abuse by the American Psychiatric Association (APA; 1994), has been described as a chronically relapsing disorder characterised by the compulsion to seek and take a drug, a loss of control in limiting its intake, and the emergence of a negative emotional state when access to the drug is prevented (Koob & Le Moal, 2005). The inability to self-regulate drug use and the emergence of a negative emotional state are considered to indicate the onset of drug addiction compared to occasional, controlled social drug use and drug abuse (Koob & Le Moal, 1997).

Drug addiction has aspects associated with both impulse control disorders and compulsive disorders (Koob & Volkow 2010). Impulse control disorders reflect the positive reinforcement associated with drug abuse in that the individual experiences a growing sense of tension or arousal and then experiences relief and pleasure following the impulsive act (APA, 1994). Compulsive disorders lead to an individual experiencing anxiety or stress prior to a compulsive repetitive behaviour and following this having feelings of relief, mimicking the negative reinforcement associated with drug addiction. The change in occasional but controlled drug use towards addiction requires a change in motivation influencing the drug use. The progression to drug addiction can be seen in the shift from positive reinforcement and impulsive behaviours where an individual is driven to take a drug due to the immediate effects of the drug, to more compulsive and negatively reinforced motivation to take drugs in an effort to remove the adverse effects of long-term drug taking (Koob & Volkow, 2010).

Addicted individuals find themselves continuing to take the drug despite knowing the negative consequences of these actions. This may indicate an important deficit in learning for demanding situations, forcing the individual to decide whether the long-term desires to extinguish

drug-taking behaviours outweigh the short-term desire to take the drug to avoid immediate negative consequences, where addictive-type behaviours lead an individual to choose the latter. This learning deficit implicates modifications in the functioning of the limbic pathways, importantly within the ventral striatum and associated regions (Everitt. Parkinson, Olmstead, Arroyo, Robledo, & Robbins, 1999; Koob, 1999; Volkow, Wang, Telang, Fowler, Logan, Childress, *et al.,* 2006; Smith, Berridge, & Aldridge, 2011). The nucleus accumbens (NAc; ventral striatum), a primary target for many drugs of abuse, has been strongly implicated in drug-stimulus associations and reward, motivated behaviour and decision-making (Singh, McDannald, Haney, Cerri, & Schoenbaum, 2010; Corbit & Balleine, 2011; Stopper & Floresco, 2011). The functional integrity of the reward system, including all aspects of the mesocorticolimbic system, is important in the ability for an individual to resist the addictive properties of a substance.

Disruption to an individual and society associated with drugs of abuse, including the costs (both monetary and otherwise) involved in monitoring, treating and preventing addiction justifies the need to investigate and develop forms of therapies. The chronic relapsing nature of drug addiction demonstrates the difficulties faced in the effort to treat addicted individuals. This is evident through the great number of patients (60-80%) treated with either psychosocial or pharmacological therapies returning to their physicians or treatment programmes within the first twelve months following onset of treatment (O'Brien & McLellan, 1996). This high reported rate of relapse challenges the efficacy of current treatment techniques for many forms of addiction and questions the benefits in the clinical use of these.

1.2 Drug statistics

Drug use and addiction rates, both worldwide and in New Zealand, are ever increasing, and continuously changing. As high as 7% of the adult population were estimated to have taken an illicit substance within the year of 2011, corresponding to between one and three hundred million people

(World Drug Report [WDR], 2013). However the prevalence rates of those with drug use disorders and dependence have not changed, with approximately 12% of those who reported drug use being classified (WDR, 2012).

The prevalence of cocaine use in Oceania (combining Australia with New Zealand), which is higher than that found in Europe and United States of America, has doubled within recent years from 1.0 per cent in 2004 to 2.1 percent in 2010 (WDR, 2013). Although cocaine appears to be less problematic than other psychostimulants and illicit substances, especially in comparison to rates of cannabis use, the lack of effective treatments for addicted persons highlights the need for further research into the drug. Global prevalence rates of cocaine use are estimated at around 0.4% (WDR, 2013), with the highest rates being found in the Americas and Oceania.

1.3 Cocaine

Cocaine hydrochloride (HCl) is a highly addictive psychostimulant with great abuse potential. The high levels of euphoria commonly associated with cocaine use act as the initial reinforcing stimulus, compelling an individual to repeat administration. With repeated use however, neuroadaptations might lead to an individual becoming addicted to the drug (Kalivas, 2007). Acutely, cocaine will generally produce a feeling of profound subjective well being and increased alertness, followed by magnification of the intensity of normal pleasures, emotions and sexual feelings, self-confidence and self-mastery are increased with reduced social inhibitions and satiation of appetite. Feelings of anxiety quickly appear after the initial euphoric effects, leading users to co-administer other drugs, such as alcohol, heroin and marijuana, to remove the strong sense of anxiety (Gawin, 1991). Extended use increases the likelihood of drug-taking behaviours being associated with the removal of the negative effects of the 'crash' (e.g., craving, depression, agitation, rapid intensification of anxiety, suspiciousness and paranoia) leading to a transition of high-dose, long-duration binge sessions where re-administration may occur every 10-30 minutes (Gawin, 1991).

Cocaine's ability to produce such strong positive and negative effects in an individual leads to a greater potential for addictive-type behaviours.

Originating from the leaf of the coca plant (Erythroxylum novogranatense) grown in the Columbian and Central American regions, cocaine was initially used by the people of the Andes Mountains much the same as chewing tobacco, in an effort to increase stamina and reduce feelings of fatigue and hunger (Freye, 2009). In 1860, cocaine was chemically isolated from the coca leaf quickly leading into scientific research, and to its use in medical and social contexts in Western cultures. In 1884, the first prescription for cocaine took place, to relieve considerable pain for a patient suffering from severe throat cancer, leading to cocaine becoming one of the most valued therapeutic agents among medical providers. Physicians began to notice other potential uses for cocaine including mood elevation, topical anaesthesia, stimulation and pain relief (Spillane, 2000). Cocaine became increasingly popular in the medical society and was commonly prescribed for the treatment of opiate and alcohol addiction, as a tonic that reinforced and stimulated mind and body, and as a treatment for sinus conditions including colds and hay fever (Spillane, 2000). By 1903, cocaine was used in many medications, tonics and wines, including the popular drink, Coca-Cola. Increasing concern of abuse potential of cocaine was raised due to the availability, popularity and large amounts of cocaine extract in these tonics. The federal Government of the US in 1906 attempted to regulate the use and abuse of cocaine, placing labels on food items containing cocaine extract (Pure Food and Drug Act, 1906) and eventually through prohibition of cocaine (Harrison Narcotic Act; Terry, 1914).

Recreationally, cocaine can be administered in many different forms, generally via intranasal, intravenous or smoked routes of administration (Fischman & Foltin 1998). Powder cocaine (cocaine HCl) can be ingested intranasally and dissolved in water to inject directly into the bloodstream, whereas crack cocaine, also called freebase, is in crystal form and can be smoked through a glass tube getting its name because when heated, the crystals begin to make a cracking

noise (Freye, 2009). Once administered, there is little difference in the pharmacokinetics of the drug and no difference in pharmacology (Hatsukami & Fischman, 1996). Rate of onset, intensity and duration of the drugs 'high' are greatly dependent on the route of administration. Smoking and injecting cocaine produce the highest concentration most rapidly, with feelings of the 'high' evident after seconds of administration (Fischman & Foltin, 1998).

In the brain, cocaine has the ability to inhibit dopamine (DA) reuptake by the dopamine transporter, with similar affinities for serotonin and norepinephrine. It is commonly accepted that the addictive potential of cocaine stems from the increasing alteration of DA transmission. Dopaminergic projections from the ventral midbrain (ventral tegmental area) to the NAc, extended amygdala system, and the prefrontal cortex (PFC) drive reward learning processes (Hyman, Malenka, & Nestler, 2006). All drugs of abuse, including cocaine, directly or indirectly promote DA transmission in the ventral midbrain pathways through binding to the dopamine transporter (DAT; Seiden, Sabol, & Ricuarte, 1993). Recent approaches to study the abuse potential and relapse properties of cocaine addiction have utilised animal models of addiction focussing on pharmacotherapies in order to stabilise normal brain function, especially within the DA system.

1.4 Animal models of addiction and relapse

Animal models are designed to develop knowledge encompassing both behavioural and neurobiological aspects of addiction. Many aspects of drug seeking, taking and craving can be modelled in rodents in the hope to gain more understanding of the neuropharmacological mechanisms of action and neuroanatomical circuits associated with the administration drugs of abuse (Koob, 2000). Animal models take the advantage of using animal subjects in controlled laboratory conditions to study many aspects of drug seeking, taking and craving, with the potential to translate any results to human patients (Panlilio & Goldberg, 2007). While physical dependence to drugs is simple to quantify in humans, addiction in animal models can be more difficult to model.

Self-administration techniques, where animals increasingly administer a drug of abuse voluntarily, present the most validated model of addiction (O'Brien & Gardner, 2005). Relapse in animal models can be defined as a forced period of abstinence from the drug and the paired environment, compared to a reinstatement model, which allows the animal to extinguish behaviour through removal of the drug in the paired environment (Reichel, Moussawi, Do, Kalivas, & See, 2011). It is important to distinguish between relapse and reinstatement, as most animal models of addiction implement an extinction-reinstatement model (Shaham, Shalev, Lu, De Wit, & Stewart, 2003), however in human treatments, there is no obvious extinguishing of drug cues.

1.5 Drug self-administration paradigm

The self-administration paradigm produces a controlled laboratory environment for the study of animals (generally rodents: rats and mice) partaking in active drug seeking and taking behaviours in order to measure levels of drug reinforcement. Operant chambers act as the drug-paired environment where animals respond (generally with a lever press or nose-poke for rodents) for a reinforcer, whether it is food or a drug of abuse. These operant chambers have the ability to be manipulated to model different forms of self-administration, with alterations in routes of administration, different forms of conditioning stimuli (stimulus lights, tones and shocks), and the potential to apply different models of reinstatement and relapse. The reinforcers are generally administered in similar routes as human use; for example, alcohol may be administered orally whereas cocaine will commonly be administered intravenously.

In the controlled laboratory environment, drug self-administration relies on the appetitive properties of addictive drugs. This is so because animals will voluntarily self-administer drugs that are addictive for humans (Gardner, 2000); that self-administration occurs in the absence of tolerance, physical dependence, withdrawal and previous self-administration and that the drug rewards seem to activate the same neural substrates that are activated through electrical stimulation

reward (Wise, 1996). Panalilio and Goldberg (2007) describe self-administration as the animal model of addiction that most closely relates to natural human addictive behaviours, providing a suitable model to test potential therapeutic treatments. Self-administration paradigms have the ability to model multiple aspects of human addiction, including the initial administration, conditioned responding, extinction, withdrawal, and relapse or reinstatement of responding for the abusive substance.

Reinforcement contingencies in self-administration experiments vary greatly; fixed-ratio (FR), variable-ratio, fixed-interval, variable-interval, and progressive-ratio (PR) have all been used (Review: Gardner, 2005). FR schedules of reinforcement are the most common in drug self-administration. In a FR schedule of reinforcement, the animal will receive access to the drug following the fixed-ratio of responses. If the schedule is a fixed-ratio-1 (FR1), the animal is administered the drug following one of the required behaviours, a fixed-ratio-3 (FR3) schedule requires three responses before administration of the drug. It has been argued that although animals working on low FR schedules (FR1) do show a desire for the drug, as the animal will press the lever for access to the drug, it is more difficult to measure the levels of reinforcement of a certain substance (O'Brien & Gardner, 2005). It is important that the model is truly reflecting addiction; therefore using a higher FR schedule is beneficial, indicating that the reinforcing effects of the drug are strong enough to ensure the animal will continue to respond until the reinforcement is administered.

Self-administration allows not only for the testing of drug-taking behaviours but also for drug craving and seeking behaviours in the form of relapse and reinstatement. A model of reinstatement generally consists of three stages: self-administration, extinction, and reinstatement (Reichel & Bevins, 2009). The self-administration stage is generally consistent between both models of reinstatement and relapse. The extinction stage follows immediately after self-administration acquisition and stabilisation, where the animal is exposed to the self-administration

chamber with no cues present (for example: stimulus light activated, sound of the infusion pump present, and the levers disabled) and no drug reward is delivered. Extinction sessions last until the subject has reduced their responding to a set percentage of their self-administration phase. Following the last extinction session, reinstatement sessions take place where the rat is exposed to the self-administration chamber with a trigger (e.g., conditioned cues, stress or the drug itself) used to generate potential drug-seeking behaviours. During the reinstatement model of relapse, withdrawal from the drug occurs during the extinction phase, in the forced abstinence model however there is no extinction of behaviours during withdrawal. Forced abstinence models begin with self-administration or drug taking acquisition as defined above, however, rather than a period of extinction, the subject is removed from the drug-paired environment for a prescribed period of time. As there is no extinguishing of behaviours, at time of relapse, the drug-taking behaviours and environmental stimuli are intact (Fuchs, Branham, & See, 2006; Reichel & Bevins, 2009). The forced abstinent model demonstrates many aspects of human withdrawal, and drug-seeking behaviours. Chronic drug users tend to experience a period of forced or voluntary abstinence before the opportunity for relapse is presented, without the potential to extinguish their drug-taking behaviours, as modelled in the reinstatement model of relapse.

There are many ways that an individual can be triggered into reinstating their old drug-taking behaviours. The drug, or other drugs of abuse, stress, and certain environments can be strong triggers for an abstinent drug-user to resume to old habitual behaviours even following an extended period of withdrawal (Shalev, Grimm, & Shaham, 2002). Drug-primed reinstatement or relapse in an animal model of addiction refers to a single, non-contingent administration of the addictive substance reinstating previous drug-taking behaviours. Research conducted by de Wit and Stewart (1981) has demonstrated the effective nature of a drug-priming injection of an addictive substance to reinstate opioid and stimulant responding in laboratory animals. The self-administration model of

drug-primed reinstatement in experimental animals is comparable to a single alcoholic drink re-establishing addictive-type behaviours apparent previously in an alcoholic individual.

Stress can also reinstate drug-taking behaviours. Stress and related negative affect behaviours are seen as triggers in both animal and human drug-seeking and drug-taking behaviours (Shiffman *et al.,* 1996). Similar to the negative reinforcing effects of the drug itself, the use of drugs to remove stress-related emotions leads to a return to habitual behaviours through drug administration. In self-administration operant chambers, stress can be triggered through electrical foot shocks produced by the grid in the chamber (Shaham & Stewart, 1995) and even a specified period of food deprivation (Shalev *et al.,* 2000).

Cue-triggered relapse, including any environmental stimulus that was previously associated with drug self-administration, is a long-known risk factor for human relapse to addictive substances. The self-administration paradigm takes advantage of previously drug-associated environmental cues, using stimulus lights, tones, and other sensory stimuli to cue drug-seeking behaviours following extinction or abstinence from the drug of abuse (Meil & See, 1997). This model of reinstatement effectively mimics the effects that drug paraphernalia and certain environments have on addicts following periods of withdrawal. In many ways, once an individual has fallen into an addicted state, they will remain at risk for relapse for the rest of their lives regardless of whether they remain abstinent, as there will always be potential triggers for relapse.

A great deal of information can be gathered through animal models of addiction using self-administration. This model effectively portrays the active seeking of drug reinforcement, therefore has high translational value for preclinical research into human addiction. As mentioned above, self-administration paradigms allow for the study of additional elements leading to addiction and relapse, including cue related responding, drug-primed cue-induced and stress-induced relapse.

1.6 Reward circuitry and the role of DA.

Multiple regions and systems of the brain have been implicated in the processes of addiction. The neurocircuitry is extensive and mediates both the positive and negative reinforcing factors associated with drugs of abuse. The mesocorticolimbic DA system has been implicated in drug reinforcement (Koob, 1992). DA in the ventral tegmental area (VTA) projects to the NAc, extended amygdala system, PFC and other forebrain regions. In the absence of drugs of abuse, the mesocorticolimbic system acts as a regulator and gating system for the limbic system in response to biological drives and motivational factors, with natural rewards increasing levels of NAc DA similar to drugs of abuse. It is well accepted that all drugs of abuse modulate the DA system.

Psychostimulant drugs, such as cocaine and methamphetamine are known to cause an increase in DA throughout the reward system due to being indirect agonists of the DA system. It is commonly accepted that cocaine's ability to act as a reinforcing agent is mainly associated with its ability to enhance DA transmission (Sora *et al.,* 2001). There is an increase in midbrain DA firing leading to increased levels of DA release in terminal regions, including the striatum and prefrontal cortex (Koob & Le Moal, 2008). Through binding to the DAT, cocaine leads to an increase in DA present in the synapses of the NAc, prolonging synaptic DA and allowing it to diffuse more effectively between the synapses (Bradberry, 2000).

It was previously thought that DA's role in addiction was due to a direct relation to the hedonic response to an addictive substance. However it has become increasingly clear that DA's role in reward is related to its ability to encode prediction of reward, imprinting incentive value to the reinforcers and the ability to facilitate reward learning associations (Volkow & Baler, 2013). This can be seen by the fact that VTA DA initially responds to the first exposure to a novel reward, however through repeated exposure, VTA DA response can be seen on the exposure to the stimuli that predict the reward (Schultz *et al.*, 1997). This change in firing demonstrates the shift of DA's

role in reward learning and memory, increasing the chance of a certain behaviour following exposure to certain stimuli.

Volkow and Baler (2013), along with others (Koob & Le Moal, 2001; Volkow *et al.,* 2011) proposed a model implicated in the urge to take drugs of abuse. The model encompasses six interacting circuits including those implicated in reward/saliency, memory/learning/habits, inhibitory control/executive functioning, motivation/drive, interoception, and aversion avoidance/stress reactivity. These interacting circuits, when disrupted, contribute to the compulsive attributes associated with the underlying effects of drug addiction. The above encompasses the reward system and the extended systems that it projects to, including the NAc (reward/salience), amygdala and hippocampus (memory/learning/habits), PFC and OFC (inhibitory control/executive functioning), dorsal striatum and motor cortex (motivation/drive), insula and anterior cingulate cortex (interoception), and the habenula and amygdala (aversion avoidance/stress reactivity).

The mesolimbic, mesostriatal, and mesocortical DA systems are crucial for drug reward, implicating the many DA receptors and the individual functions the individual systems perform. DA D1-like receptors (including D1 and D5 receptors) when activated lead to an increase in intracellular levels of cyclic-AMP (cAMP). The D2-like receptors (including D2, D3, and D4 receptors) are negatively associated to the production of cAMP (Chen & Xu, 2010). D1 and D3 receptors can both be found largely in the NAc, among other areas of the brain, and have been found to mediate the locomotor and positive reinforcing effects of cocaine as well as the cue-induced reinstatement of seeking for cocaine (Liu *et al.,* 2009). Drug-induced DA signalling triggers neuroadaptations, in circuits related to habit formation and behavioural conditioning, largely induced by D1 receptor activation (Luscher & Malenka, 2011). D3 receptors, which found almost exclusively in the shell of the NAc, have agonists with powerful effects in supressing self-administration of cocaine in the rodent (Caine *et al.,* 1997). Increases in D1 receptor signalling

leads to an increase in drug reward, whilst the opposite effect can be seen in D2 receptors with a reduction in reward associated with enhanced signalling of the receptor (Lobo & Nestler, 2011).

Reduction in striatal D2 receptor levels has been found in many preclinical trials with repeated exposure to drugs of abuse (Nader *et al.,* 2006; Thanos *et al.,* 2007; Volkow *et al.,* 2001). In the striatum, D2 receptors have been shown to mediate the signalling of the striatal indirect pathway modulating the PFC, and in animal models its downregulation has been seen to enhance sensitisation of the effects to drugs (Ferguson *et al.,* 2011). In humans, low levels of striatal D2 receptors are associated with decreased activity in the PFC, which is involved in salience attribution, inhibitory control, emotional regulation and decision-making (Volkow *et al.,* 2000). Essentially, these low levels of DA D2 receptor signalling are associated with impulsive behaviour and appear to result in enhanced motivational value of a drug and the loss of control over drug taking (Belin & Everitt, 2008). D2 receptor function is increasingly important in the study of addiction as it has been shown that impaired modulation in OFC and striatal regions in rodents leads to increase obsessive compulsive behaviours and predicted compulsive drug taking (Volkow and Fowler, 2000; Everitt *et al.,* 2008). Irregular functioning of the D2 receptors within the striatum has been seen to predict whether a naïve individual will find an abusive substance aversive (high levels of D2 receptor) or pleasurable (low levels of D2 receptor) (Volkow *et al.,* 1999, 2002).

The above circuitry is initially connected through DA innervations, however they are also connected through both direct and indirect projections, mostly glutamatergic and modulated by other monoamines such as 5HT, NE and GABAergic projections. This implication of other neural elements reflect the multidimensional nature of drug addiction and the reasons that it may overlap with other psychiatric disorders (Volkow and Baler, 2013). There are extensive afferent projections of glutamate to DA neurons from regions associated with sensory, homeostatic, reward, emotional, and multimodal information processing modulating the DA response to rewards and conditioned cues (Geisler and Wise, 2008). Synaptic plasticity is controlled by the presynaptic release of

glutamate and the post-synaptic insertion or removal of AMPA or NMDA glutamate receptors; drugs of abuse interfere with both of these processes (Brown *et al.,* 2011; Huang *et al.,* 2009; Koya *et al.,* 2012). Both AMPA and NMDA receptors are increased following cocaine withdrawal and chronic exposure to cocaine respectively (Boudreau *et al.,* 2007; Huang *et al.,* 2009).

Allostasis, achieving stability or homeostasis through physiological or behavioural change, seeks to return an individual to internal stability (McEwen, 2000; Koob & Le Moal, 2008). When drugs and addiction are involved, allostatic load can occur due to the recurring deviations from homeostasis. The points of allostasis after repeated deviations from the use of abusive drugs continually decreases, leading an individual to fail to return to normal rates of homeostasis. Solomon (1980) proposed that there are two opponent processes, the a- and b-processes involved with reward. The a-process occurs immediately following the presentation of a stimulus, matching the intensity, quality and duration of the reward, with tolerance occurring over time. The b-process occurs after the termination of the a-process. The b-process has a significantly slower onset, is slower to reach full strength and to decay, and its effects increase with repeated exposure. In terms of addiction, the a-process relates to the initial euphoric response to the drug, and the b-process relates to the negative effects and craving following the initial positive effects. Increases in the b-process, due to the adverse effects of craving and withdrawal, lead an individual to attempt to maintain the euphoric effects of the a-process. This leads to changes in the brains molecular, cellular, and neurocircuitry properties in order to maintain a level of stability (Solomon & Corbit, 1974). The combination of increases of reward and the other important factors important in addiction (decreased functionality or reward systems, use of anti-reward systems, loss of executive control, and stimulus-response associations) lead to the compulsive behaviours associated with drug seeking and taking (Koob & Le Moal, 2008).

1.7 The nucleus accumbens.

The reinforcing effects of a psychostimulant depend largely on the mesocorticolimbic DA systems activating the NAc (Wise, 1981). Crucial to the rewarding effects of abusive drugs is the projection of DA cells within the VTA to the NAc (Wise, 2009). When stimulated by DA, the cells of the NAc generate feelings of pleasure and satisfaction, and in non-drug activation, this helps to keep an individual focussed on the basic biological goals of survival and reproduction (Nestler, 2005). Herrick (1926) originally linked the NAc to motivational processes through the suggestion that it was involved in locomotion and feeding. Research surrounding the NAc has since developed on this idea to work surrounding reinforcing effects of both natural and drug rewards. DA is a critical element in the goal-directed behaviours and incentive salience associated with the NAc, however it is clear that in terms of conditioned behaviour there is more at play. Glutamate and changes in its transmission within the NAc appears to produce the plasticity that triggers addiction (Kelley *et al.,* 2003). Everitt and Wolf (2002) discuss the importance that plasticity in neural systems has in the promotion of addiction, especially that of the NAc and the dorsal striatum. Within the NAc, the consolidation of behaviours due to the reinforcing effects of a psychostimulant may be the final progression to addiction where control over drug use is lost and craving takes over control (Everitt *et al.,* 2001; Hyman & Malenka, 2001).

The striatum is divided into two major subregions, the dorsal and ventral striatum. The dorsal striatum consists of the caudate-putamen, and the ventral region consists of the NAc. The NAc, which can be further divided into the core and shell, is involved in adaptive and goal directed behaviours. The shell and core differ in morphology due to the location of DA afferents. The shell receives DA projections solely from the VTA, whereas the core receives DA projections both from the VTA and the substantia nigra (SN) (Brog *et al.,* 1993). The subdivision has been demonstrated through work in behavioural, electrophysiological, and neurochemical research revealing that there

are differing motivated behaviours between the two (Kelley & Swanson, 1997; Stratford & Kelley, 1997; Barrot *et al.,* 2002).

Kelley and colleagues demonstrated the neuropharmacological differences between the 'central core' and the medial shell of the NAc. The NMDA-receptor antagonist (AP-5), decreased locomotor activity, rearing, and novel object exploration following injections into in the core but not into the shell of the NAc (Maldonado-Irizarry & Kelly, 1994). This evidence was reproduced and supported by Pulvirenti and colleagues (1994) who showed attenuation of cocaine induced hyperactivity through administration of AP-5 in the core but not the shell. In contrast, the shell of the NAc is more associated with the unconditioned response to drugs of abuse. There is evidence that the unconditioned DA response to morphine and cocaine is significantly higher in the shell than the core (Pontieri *et al.,* 1995). Everitt and colleagues (1999) demonstrated that the neural network necessary to mediate the stimulant effects of drugs lies in the shell of the NAc, as the magnitude of DA response for psychostimulant drugs is reliant on an intact shell. More recently there has been evidence that the shell of the NAc is important for the reinstatement of drug-seeking behaviours (Anderson *et al.,* 2003, 2006; Schmidt *et al.,* 2006, Schmidt & Pierce, 2006). Developing on previous evidence, Vassoler and colleagues (2008) concluded that disruption of neuronal activity in the shell might attenuate drug-primed cocaine reinstatement. Although it is clear that both core and shell are key regions in the processing of reward-related information and both function as an interface between the limbic and motor systems, they show distinct neurochemical and anatomical characteristics (Heimer et al., 1991, Lanca et al., 1998, Pickel et al., 2001), which have functional significance with regards to their response to drugs and mediation of drug effects.

1.8 Treatments

They key feature of addiction is the inability of addicts to prevent relapse. This feature denotes the need to generate efficient and effective forms of treatment, whether they are

pharmacological or therapy based. In stimulant addiction, pharmacological treatments have been developed and are aimed at blocking the actions of a certain drug or acting as a substitute for them. Research into treatments is yet to find a suitable treatment to assist in all aspects including, detoxification, withdrawal, and relapse prevention. With recent progress in the understanding of the reward system and regions associated with all aspects of drug abuse, there has been development of some novel forms of treatment. One such treatment is deep brain stimulation (DBS), which has been successfully used in the treatment of Parkinson's disease (PD) symptoms (Wichmann & Delong, 2006).

1.8.1 Deep brain stimulation DBS is a technique where electrodes are implanted in a target region in the brain to deliver micro-electrical pulses. Conceptually, it is a similar procedure to ablation or electrical activation of a target region (Halpern *et al.,* 2007). DBS has many functional advantages over surgical ablation, as it is considered a less invasive, reversible and adjustable procedure. When applied, the stimulation parameters can be adjusted to remove any unwanted side effects, allowing the procedure to be tailored to each individual patient or participant's requirements.

DBS has been used as a treatment for neuropsychiatric disorders, such as obsessive-compulsive disorder (OCD) and refractory movement disorders such as PD (Wichmann & Delong, 2006). DBS of thalamic and subthalamic nuclei have emerged as effective treatments for a number of psychiatric patients who fail to respond to current pharmacological or behavioural therapies including patients with PD, dystonia and tremor, Tourette's syndrome, and depression (review: Wichmann & Delong, 2006).

DBS has also been postulated as an effective treatment for treatment-refractory drug addiction, although it is not yet clear which areas of the brain are the most effective as targets for the reduction of symptoms and to protect against relapse (Luigjes *et al.,* 2011). The NAc has

recently been proposed as a promising target for DBS treatment for substance addiction-related disorders (Kuhn et al., 2007, Liu et al., 2008, Vassoler et al., 2008, Knapp et al., 2009, Kuhn et al., 2009, Muller et al., 2009, Henderson et al., 2010, Mantione et al., 2010, Zhou et al., 2011). Some limited animal and human research has explored the effectiveness of NAc DBS in reducing drug-related behaviours and symptoms, as applied to drugs including alcohol (Knapp, Tozier, Pak, Ciraulo, & Kornetsky, 2009; Muller *et al.* 2009; Kuhn *et al.* 2007; Henderson, *et al.* 2010), cocaine (Vassoler *et al.* 2008), nicotine (Kuhn *et al.* 2009; Mantione, van de Brink, Schuurman, & Denys, 2010), and opiate addiction (Liu, *et al.* 2008; Zhou, Xu, & Jiang, 2011). This consistent evidence, although still limited, offers support for the NAc as a critical region in the mediation of drug-related behaviours, and as a potential target for the therapeutic potential of DBS treatment in addiction.

The NAc consists of two distinct subregions and a number of studies using DBS have reported the differing effects generated through stimulation of either the core or the shell of the NAc. Sesia and colleagues (2010) provided evidence of this, where they stimulated both areas in animals and demonstrated that DBS in the NAc shell decreased DA and 5HT turnover, whereas DBS of the NAc core did not. Moreover, stimulation of the shell generated greater levels of impulsivity where stimulation within the core did not. Vassoler and colleagues (2008, 2013) also demonstrated the differences in stimulating the core and shell using a reinstatement model of self-administration. They report evidence that the shell but not the core reduced cocaine-primed reinstatement of cocaine seeking following extinction. This preliminary evidence, although still limited, demonstrates site-specific effects of DBS within the NAc in relation to drug-related behaviours.

The mechanisms underlying the effects of NAc DBS treatment are largely unknown. Some hypotheses of mechanisms of action include the increase of activation locally in the target region (McIntyre *et al.,* 2004; Montgomery & Gale, 2008). In comparison, others suggest that DBS leads to inhibition through the depolarisation blockade or through activation of inhibitory neurons

(Boraud *et al.,* 1996; Banazzouz and Hallet, 2000; Kiss *et al.,* 2002). One working hypothesis elaborates further on the above and states that the stimulation may activate afferent or efferent pathways leading to distant synaptic inhibition or excitation and modulation in dysfunctional networks (Luigjes *et al.,* 2011). This may occur through antidromic stimulation of the afferent cortical projections, and potential activation of the basal ganglia thalamocortical network. Vassoler and colleagues (2013) report evidence that stimulation of the NAc shell attenuated drug reinstatement through local activation and GABAergic interneuron activation in the prefrontal cortex, a result of antidromic stimulation of cortico-accumbal afferents. The orbitofrontal cortex has been implicated again, with dysregulation of OFC and its dense NAc projections leading to mediation of drug craving and compulsive drug taking (Volkow & Fowler, 2000). In turn, the NAc projects back through the mediodorsal nucleus of the thalamus to the OFC (Ray & Price, 1993), implicating a connected network of regions implicated by the stimulation of one target region, the NAc. Prolonged accumbens stimulation has been shown to produce long-term potentiation in cortical interneurons, potentially contributing to a long-term effect of DBS (McCracken & Grace, 2007). Current research is implicating both local and axonal activation/inhibition generating the idea that both hypotheses have potential, leading to the requirement of new studies in hope of determining the exact mechanisms underlying the positive effects of DBS.

There are many important variables when considering the therapeutic potential of DBS for the treatment of drug addiction. The most important, following determination of the target regions, is the appropriate stimulation parameters required for an individual and the specific symptoms to be treated. The majority of previous studies concerned with DBS typically use high frequency (HF) stimulation (130-160 Hz), where the beneficial effects are likely to be due to a functional inhibition of the stimulated areas. In most DBS studies, the parameters are fixed throughout, generally using biphasic, symmetrical pulses (60 μs pulse width) with HF stimulation (160 Hz). Stimulation intensities however are not quite as consistent throughout the field, with a range from 50-200 μA

(Chang *et al.,* 2003, Mayberg *et al.,* 2005). Vassoler and colleagues (2008, 2013) presented evidence in support of 150 μA producing an effective intensity for their drug reinstatement paradigm in rats. The success of DBS for the treatment of addiction is dependent on the frequency (Hz), pulse width (μs) and amplitude (μA) parameters chosen by the researchers. Dependent on the target region in the brain, frequency of the stimulation relates to functional activation or inhibition of the region and any afferent and efferent manipulations. In the NAc, it is believed that HF (130-160 Hz) results in inhibition and low frequency (LF; 5-20 Hz) results in activation of the corresponding neurons.

The aim of DBS treatment is to reduce the symptoms of a neuropsychiatric disorder while producing minimal disruption to neural circuits, therefore any non-specific damage due to stimulation needs to be kept at a minimum. Pulse width is related to the amount of tissue damage following stimulation; reduced pulse widths result in the probability of inducing less damage. Amplitude, referring to the magnitude of the pulse, determines the levels of activation in the surrounding tissue. If the amplitude is set too high unwanted side effects can appear and if it is too low, the desired effects of stimulation may not occur. Amplitude is not a consistent parameter that can be set over a population. Individuals need to be examined in the initial DBS treatment, with the amplitude being altered to remove any unwanted side effects (Kuncel & Grill, 2004). Biphasic or monophasic wavelength stimulation has an important impact on the tissue damage that may occur, especially at HF stimulation levels. Monophasic pulses induce a current that flows in only one direction that goes directly into the brain, whereas biphasic pulses generate a current that initially goes into the brain and then a reverse current. This reverse in direction minimises the accumulation of charge at the tip of the electrode (Gubellini *et al.,* 2009). Monophasic pulses, due to the accumulation of charge at the tip of the electrode, have been linked to greater levels of tissue damage. Therefore, use of biphasic pulse wavelengths is recommended to produce a safer and more effective DBS treatment for addicted individuals (Merril *et al.,* 2005).

Bilateral implantation of electrodes into the target regions in the brain has become common practice for clinical patients receiving DBS therapy and within animal research in neuropsychiatric disorders, including addiction disorders. Alberts and colleagues (2008) raised the argument that although it is common practice and that there are generally significant results gained, bilateral implantations lead to greater complication such as post-operative confusion, speech difficulties, and cognitive dysfunction. Unilateral implanting has been shown to greatly reduce these potential side effects, and unless a patient requires the bilateral implant, patients should be able to avoid the potential dangers and receive unilateral electrodes. It is important to develop on the knowledge within the DBS field as to whether the same benefits from bilateral DBS can be produced with less invasive, unilateral electrode implantations.

Although DBS has been proposed as a safer and less invasive form of surgical ablation and lesions, it is still important to understand that implantation of electrodes into the brain is considered major surgery. When considering DBS for human use, it is important to understand that the stereotaxic procedure to implant any electrode into a brain region is expensive, invasive and potentially damaging to the brain. The stimulation parameters once implanted are reversible, whereas any damage to surrounding brain tissue made during surgery may have lasting effects implicating the individuals' everyday functioning. The potential issues related to the surgical implantation of an electrode into a target brain region leads to the requirement of criteria to elucidate which patients are truly in need of this treatment. It is important to determine that the individual is treatment resistant, that is, that the patient has experienced full advantage of all other non-invasive forms of treatment including rehabilitation clinics, behavioural, pharmacological, and cognitive therapies. Hall and Carter (2011) discuss the importance for determining treatment resistance through the fact that some patients will fail to meet the criteria purely due to lack of resources. With the potential for infections and cognitive, behavioural and emotional instabilities along with possible psychosocial changes from the insertion of an electrode in the brain (Synofzik

& Schlaepher, 2011; Kleiner-Fisman *et al.,* 2006), full consent needs to be paired with an outline of all the risks involved with stereotaxic procedures.

There are many unanswered questions about DBS stimulation, especially in relation to addiction behaviours. One of these issues related to the idea that DBS is an invasive technique that may produce short-term or long-term aversive disturbances to behaviour, emotion, and cognitive functioning even if the stimulation is deemed successful (Synofzik & Schlaepher, 2011). This justifies the need for testing situations that observe both acute and long-term adaptive changes in the brain following surgical implantation. Another issue, surrounding the previously mentioned stimulation parameter, is whether HF or LF stimulation is more effective and should be applied for the therapeutic DBS treatment of addiction. Most animal studies have examined the effects of HF stimulation, whereas LF stimulation is generally used in clinical treatments, as it is safer for use in human patients (Anderson *et al.,* 2006). Finally, it is important to determine exactly when and how long the stimulation should be applied. Most studies in DBS for the treatment of addiction have applied stimulation during the test session (relapse; Henderson *et al.,* 2010; Vassoler *et al.,* 2008, 2013; Knapp, Tozier, Pak, Ciraulo, & Kornetsky, 2009; Liu *et al.,* 2008; Rouaud *et al.,* 2010), thus introducing a potential confound, where the stimulation itself could interfere with the drug-related behaviour or the drug-seeking task. It is necessary to include evidence of stimulation applied prior to testing sessions to ensure the effects are similar to previous studies with stimulation applied during testing sessions and that this model does not generate confounds. It is important to note that clinical studies using prolonged and continuous DBS in the NAc of patients is neither reinforcing nor aversive (Sturm *et al.,* 2003; Kuhn *et al.,* 2007; Schlaepfer *et al.,* 2008) therefore DBS alone will not promote drug taking or seeking or cause adverse effects for the patient.

There is very limited evidence from clinical use of DBS in human addiction patients. Most clinical evidence is from patients following treatment for other disorders such as PD or OCD (Carter, Bell, Racine, & Hall, 2011). While there have been some promising results, it is premature

to conclude that DBS is an effective treatment for addiction related behaviours. It is important to continue with further preclinical animal research into the potential effects of DBS on addiction related behaviours and to determine the most effective brain regions to target for the reduction of unwanted behaviours, and the long-term protective effects of stimulation on cocaine craving and relapse.

2.0 Rationale, aims and hypotheses

Drug addiction, the inability to self-regulate drug use and the onset of a negative emotional state, poses an enormous burden on society, as there remains an unmet need for prevention, treatment and care for those who suffer from addiction. The stimulation of deep brain regions (DBS) that are affected by disease appears as a novel form of treatment for neuropsychiatric disorders. Patients suffering from PD benefit from the treatment with DBS therapy, with dramatic reductions in tremor symptoms. It has recently been postulated that DBS may also provide relief to patients who suffer from intractable psychopathologies, including treatment resistant depression and drug addiction. The hypothesis is that application of a current to certain areas within the reward circuit (LF stimulation to activate and HF to deactivate) may re-establish the 'normal' functioning of circuits affected by drug abuse. In the case of addiction, it is believed that dopamine-modulated cortical and subcortical regions, encompassing the NAc, represent potential targets for stimulation therapy. The specific locations, optimal for stimulation, however, have not been identified within these relatively large and heterogeneous locations.

In an attempt to fill the gaps in knowledge surrounding DBS treatment for addiction, the proposed project aimed to investigate the potential therapeutic application of differing stimulation parameters (LF and HF) to the NAc in a well-established model of drug addiction. It is important to determine the optimal parameters for therapy, therefore the study of both LF and HF DBS will help

to obtain the information required to determine which parameters are effective for cocaine-experienced animals following periods of forced-abstinence.

Examining the NAc as a target region for DBS in the acute and long-term relief of relapse following chronic cocaine exposure is a novel approach. Building on past research into DBS therapy for drug addiction (review: Luigjes *et al.,* 2011), we aim to characterise the possible long-term anti-relapse protection properties of chronic exposure to DBS (LF and HF) in animals experiencing a forced abstinence period following chronic cocaine exposure. Ewing and Grace (2013) discuss the notable lack of research into long-term exposure to DBS despite the knowledge that chronic exposure produces differing results than acute exposure. In the present experiment we will use HF and LF stimulation to modulate the activity within the NAc and the affiliated pathways in the hope to offer long-term protection against relapse. To reduce the invasive nature of stereotaxic surgery, we have implanted unilateral electrodes rather than bilateral to determine whether the beneficial effects from DBS treatment are still apparent with a single stimulating site (Bossert *et al.,* 2012).

It was hypothesised that chronic LF and HF stimulation would reduce cocaine relapse following a period of forced-abstinence. A greater effect was expected from those animals treated with HF stimulation, due to the similarity between HF stimulation and lesions of the NAc, where there is inhibition in the neuronal output from the NAc and inactivation of accumbal afferents (Vassoler *et al.,* 2008) therefore attenuating cocaine seeking behaviours.

3.0 Methods

3.1 Subjects

Subjects were 40, 10-12 week-old male Long Evans rats obtained from the Animal Facility of the Department of Psychology, University of Canterbury. The animals were housed individually in polycarbonate cages (48x28x22 cm) on a reversed 12 hour light-dark cycle (lights on at 8.00PM), with standard humidity (50±5%), and temperature conditions (21±2°C). The animals were on a moderately restricted diet of 20g/day of rat chow to maintain a steady body weight, with close monitoring to ensure maintenance of the initial body weight (no substantial loss or gain in a short period of time). Water was available to the animals *ad libitum*. All procedures carried out were in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and approved by the Animal Ethics Committee of the University of Canterbury (protocol 2012/35R; Appendix A).

3.2 Pharmacological treatments

Cocaine HCl (National Institute of Drug Abuse, NIH, USA) was used for the self-administration experiment. Cocaine was delivered to the animals intravenously with each infusion administering 0.5mg/kg. The cocaine was dissolved in 0.9% saline at a volume of 1.33 mg/ml

3.3 Apparatus

3.3.1 Self-administration chambers Operant self-administration chambers (Panlab S.L., Barcelona, Spain) enclosed within sound-attenuating chambers were used to train and test the animals for cocaine self-administration and relapse. The chambers were fitted with two metal response levers, serving as the active (right) and inactive (left) levers. An active lever response resulted in reinforcement for the animal, an intravenous infusion of cocaine, from a pump located

outside of the attenuation chamber, and the activation of a stimulus light (4 cm diameter) located directly above the active lever. An inactive lever response was programmed to have no consequences. The chambers were equipped with a 4x4 cm food/water receptacle located directly between the two levers, and a general house light located in the attenuation chamber.

A) B)

Figure 1. Images of the self-administration chambers; A) self-administration, sound attenuating chambers and infusion pumps B) active (right) and inactive (left) response levers.

3.3.2 Deep Brain Stimulation Chambers DBS procedures were conducted in operant chambers (Med Associates, VT, USA) enclosed in sound attenuating chambers. These chambers are visually different to the self-administration chambers and have the opposite orientation to reduce the chances of inducing extinction responses or interference with the self-administration procedures. The DBS chambers were fitted only with a general house light and a swivel connected to the external dual channel stimulator (Grass Technology, Model S88, RI, USA), a voltmeter, and an oscilloscope to measure the frequency and amplitude of the stimulation. The house light, positioned directly in the centre of the right wall, was illuminated during the entirety of the session. The leads

in the swivel connected to the electrode that had been previously implanted in the animal, and the oscilloscope was used to ensure that the biphasic pulses generated through the stimulator were effectively entering the brain.

A) B)

Figure 2. Deep brain stimulation setup; A) leads that connect the animal to the stimulator, B) sound attenuating and modified operant chamber, oscilloscope, and stimulator.

3.3.3 Stimulation parameters Based on previous NAc stimulation studies, we used both HF (160 Hz) and LF (20 Hz) stimulation. The stimulation consisted of continuous alternating current with biphasic square. Stimulation parameters were set at a pulse width of 100 μs, and a delay between positive and negative pulses of 20 μs. The amplitude of the stimulation was adjusted individually before the main experiment to control for any unwanted, abnormal behaviours such as freezing, vocalising or jumping that would be consistent with fear, pain or other types of discomfort. Expected intensity is between 50-200 μA (aiming for around 150 μA) according to previous DBS NAc studies.

3.4 Surgical procedures

Procedures were as described previously (Ferragud *et al.,* 2009; Velazquez-Sanchez *et al.,* 2010, 2013). The animals were pre-treated with Cephalexin (50 mg/kg s.c; an antibiotic used to help

survivability of surgery) for four days prior to surgery, and exposed to the self-administration operant chambers in 15-minute sessions for those four days. During exposure, both the active and inactive levers of the chambers were removed, in an effort to habituate the animals whilst removing a possible effect of latent inhibition for later lever pressing to receive cocaine.

Prior to surgical procedures, the animals were administered Ketamine (85 mg/kg) plus Domitor (0.35 mg/kg) through intra-peritoneal injection (i.p) to induce deep anaesthesia. Carprofen (5 mg/kg) and Hartman's solution (1.0 ml) were also administered through i.p injections for pain relief and to ensure the animals remained hydrated throughout the surgical procedures. Sterile catheters (O/D 0.63 mm, I/D 0.30 mm, Camcaths, Cambridge, UK) were implanted into the isolated right jugular vein of each animal and the tubing was secured in place through the use of sutures. The mesh collar and thread tip of the catheter were implanted dorsally between the scapulae of the animal, after the tubing had been pushed through the skin to exit in the dorsal opening. The mesh collar was sutured in place and the exposed thread was sealed with a plastic cap.

Following the catheter placement, the animals were immediately mounted into a stereotaxic apparatus in order to place an electrode into the NAc. Bipolar, two channel stainless steel electrodes (diameter 0.28 mm, length 12 mm; Plastics One, VA, USA) were implanted unilaterally (right hemisphere) in the NAc according to these coordinates relative to bregma: AP +1.3 mm, ML -1.4 mm, DV -7.1 mm from brain surface (Figure 3). The electrodes were secured in place through the use of two stainless steel mounting screws (Plastics One, VA, USA) fastened to the skull to aid in holding the dental acrylic in place surrounding the remaining exposed parts of the electrode. Dust caps were fastened to the insulated end of the electrode to reduce any chance of damage to the electrode.

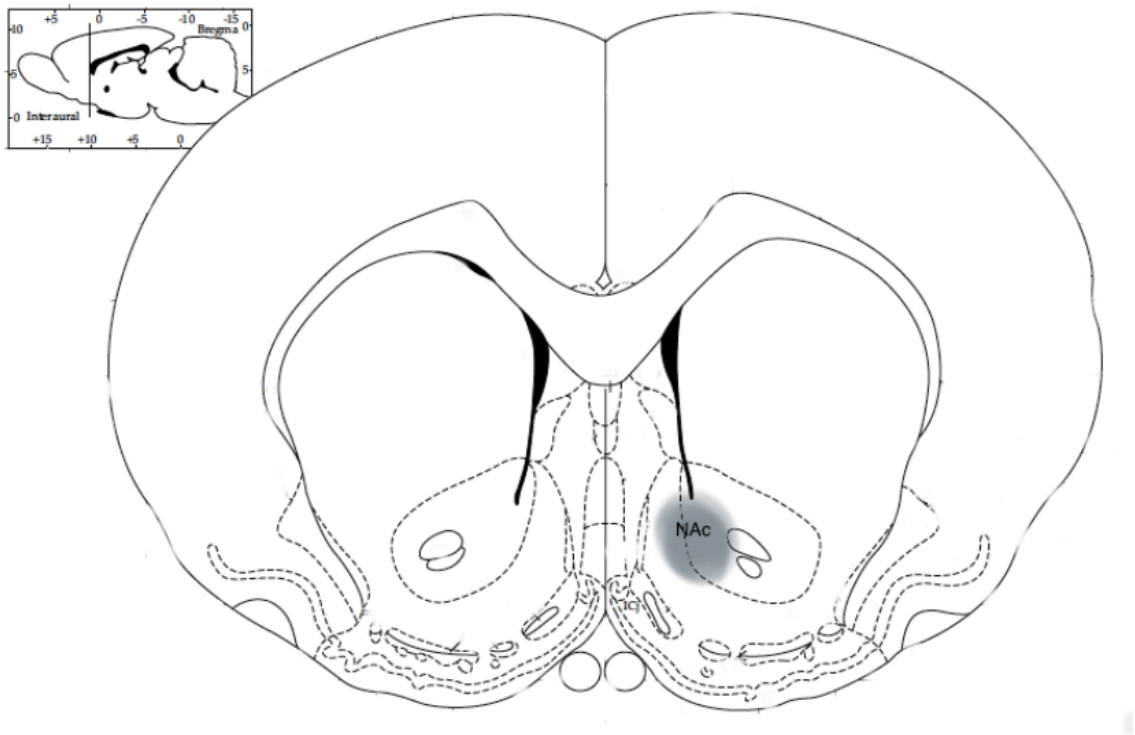

Figure 3. NAc target for DBS (AP: +1.2 from Bregma)

Immediately following the electrode implantation, Antisedan (1.75 mg/kg, i.p) was administered to reverse the effects of anaesthesia. The animals were treated with Cephalexin (50 mg/kg s.c) and Hartman's solution. Heparin (0.1 ml) was administered daily through the catheters to remove any blockages that may have occurred during the recovery period. The animals were allowed seven days recovery with food and water *ad libitum*, and continued to be housed individually before commencing self-administration pre-training.

3.5 Self-administration and deep brain stimulation

3.5.1 Experimental design

Table 1:

Experimental Design for Nucleus Accumbens Self-Administration Procedures

	Pre-training	Training 5days	DBS/Withdrawal 14days	Relapse Day1,15&30
Co-LF	FR1, FR2, FR3	Cocaine	Low Frequency	Cocaine
Co-HF	FR1, FR2, FR3	Cocaine	High Frequency	Cocaine
Co-Sham	FR1, FR2, FR3	Cocaine	Sham	Cocaine
SA-Sham	FR1, FR2, FR3	Saline	Sham	Saline

Table 1: Co = cocaine hydrochloride administered during S-A training and relapse; LF = low frequency stimulation; HF = high frequency stimulation; Sham = animals not receiving stimulation but still undergoing the same DBS procedure; SA = saline administered during S-A training and relapse. Animals were randomly assigned to one of the 4 experimental groups (Co-HF n = 8, Co-LF n = 9, Co-Sham n = 7, SA-Sham n = 6). All animals were initially pre-trained on FR1, FR2, and FR3 extended sessions to press the active lever (biased to the right) for cocaine. FR3 responding was stabilised over 5 consecutive days (training) for both cocaine and saline groups, with 4 days of no less than 20% variance. DBS occurred for the 14 days immediately following self-administration training, with the animals experiencing withdrawal conditions (no exposure to self-administration operant chambers). Relapse involved both context and drug-induced reinstatement (i.p administration) of saline or cocaine or all three sessions (days 1, 15 and 30 of DBS/withdrawal stage). Training and relapse sessions lasted for 90 minutes and DBS sessions occurred over 30 minutes.

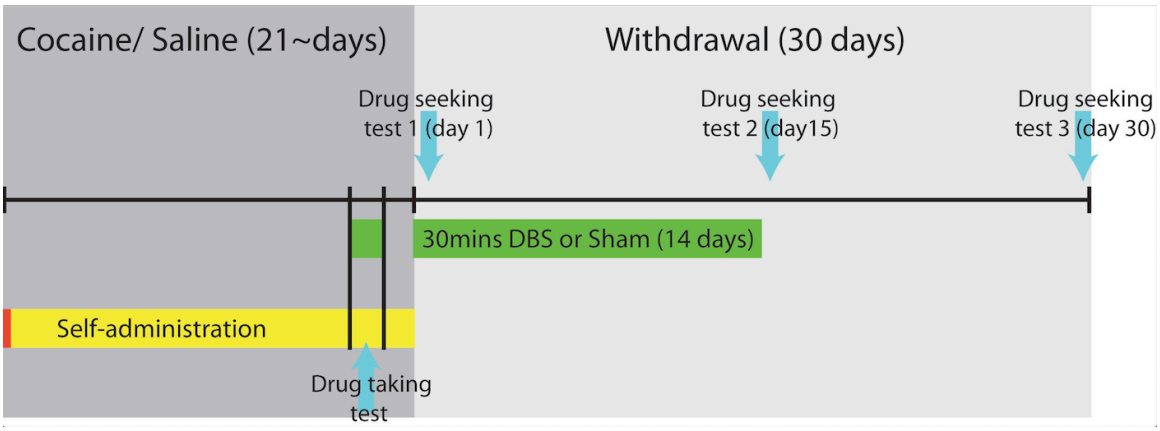

Figure 4. Schematic timeline of self-administration on withdrawal/stimulation procedures

Figure 4: Following recovery from surgery, the animals were trained for self-administration of either cocaine or saline (FR1, FR2, and FR3). At least five stable days of responding to FR3 was required to proceed into the intake test. Self-administration + DBS was required to test whether or not the responding behaviours and intake of either cocaine or saline was not disturbed by the DBS procedure. The animals were given access to the drug during the intake test (drug taking test). When responding was stable, the animal moved into the withdrawal phase, with the first day involving both a 30 min DBS session and a 90 min relapse test. The next 13 days involved one 30 min DBS session per day, with DBS occurring for 14 consecutive days (Monday through Sunday) following self-administration training. The second relapse test occurred on day 15 and the third on day 30 of the withdrawal period. These tests involved a 90 min relapse session with no drug available through the infusion pump. Between relapse test 2 and 3, the animals remained undisturbed in the holding room

3.6 Behavioural assays

3.6.1 Self-administration training Self-administration procedures, for either cocaine and saline, consisted of pre-training, training, DBS plus intake test, withdrawal and relapse phases. The PackWin software programme (Panlab, S.L., Barcelona, Spain) recorded both the active (right) and inactive (left) lever presses, along with the total number of reinforcements for each session.

During pre-training sessions, the animals were allowed extended sessions (approximately 15 hrs.) in order learn to respond to the active lever for a cocaine reinforcement on a Fixed Ratio 1 (FR1) schedule (one active lever press resulting in one infusion of cocaine). Following each reinforcement, a five second time-out was introduced where any extra active lever pressing did not

result in any reinforcement of either the drug or light stimulus. The number of lever presses during this time were still recorded on PackWin. The time-out was introduced to prevent the accumulation of multiple cocaine infusions in a short period of time. Following two stable extended sessions, with at least 30 reinforcements per session, the animals proceeded to a FR2 schedule where two active lever presses were required for reinforcement. The criterion remained at two stable sessions with at least 30 reinforcements per session to proceed to a FR3 extended session, where three active lever presses resulted in reinforcement. Pre-training concluded once the animals met the criterion of 30 reinforcements per session for the two final sessions.

3.6.2 Self-administration intake tests. Self-administration training for cocaine or saline consisted of 90 min FR3 sessions, with five stable days of responding on the active lever required. A minimum of 20 reinforcements, and less than 20% variation in the number of reinforcements were required to complete the training phase. The initial DBS session and intake test were conducted the day following the completion of self-administration training. The animals were exposed to either sham, LF or HF stimulation for half an hour and then placed into the self-administration chambers for a FR3 90 min session, where either cocaine or saline were present. The animals were allowed as many sessions following the intake test as required to restabilise their responding in self-administration, so as to produce similar responding as seen prior to exposure to DBS.

3.6.3 Self-administration withdrawal and relapse tests. Following the DBS and intake test, the animals received 14 consecutive days of sham, LF or HF DBS. All animals were monitored for their first introduction to stimulation in the DBS chamber to ensure no abnormal behaviours were elicited. Day one of the DBS phase consisted of both stimulation and a relapse test, with the animals receiving 30 min of the appropriate stimulation followed immediately by a 90 min session in the

self-administration chambers. Relapse test one involved each animal receiving a priming injection (i.p) of either cocaine (5 mg/kg) or saline immediately prior to being placed in the self-administration chamber to model a 'natural' scenario where an addict is presented with context and drug cues simultaneously (Shalev, Grimm, & Shaham, 2002). The relapse tests lasted for 90 min, the same as a training session, however the infusion pumps were disabled removing the availability of the drug in response to lever pressing. Following the first relapse test, each group continued to receive the assigned form of stimulation for a 30 min session per day, to complete the 14 consecutive days of stimulation (Monday through Sunday). Relapse test two occurred on day 15 with each animal receiving the same procedure as test one, with the only difference being that no stimulation was present on this testing day. The animals were exposed to an extended period of forced-abstinence following test two while remaining in the holding room, with relapse test three occurring on day 30 of the DBS phase.

3.7 Operant training for food reward

3.7.1 Training phase. Six animals were assigned to the saccharin (0.1 %) training group. The animals began on an FR1 extended session, where one response would result in activation of the saccharin pump releasing five drops of saccharin into the feeder. The criterion to complete the FR1 extended session phase was the same as the cocaine criteria, which is 40 reinforcements before the end of the session. On completion of the FR1 sessions, the animals began training on FR3 extended sessions, with 3 lever presses resulting in pump activation for a reward. Two stable (less than 20% variability) extended sessions of FR3 responding allowed the animals to progress into a 90 min session of FR3 responding for saccharin. Five stable days of responding to a FR3 90 min schedule were required to end the training phase.

3.7.2 Testing phase. Using a within-subjects design, each animal was tested for saccharin intake after either HF or LF stimulation. The animals were randomly assigned to HF or LF groups using within-subjects counterbalanced design for test 1 and 2, receiving 30 min of stimulation immediately before completing a 90 min intake test where saccharin was available after each lever press (FR3 schedule). Between tests 1 and 2, the animals received two days of FR3 responding for saccharin to ensure a return to stable responding before the next intake test.

3.8 Histology

3.8.1 Perfusions. After the final relapse test (day 30; 30 min following the relapse test), the animals were perfused in order to check the electrode placement. The animals were deeply anaesthetised with pentobarbital (150 mg/kg) and perfused transcardially with paraformaldehyde (PFA 4% in 0.1M phosphate buffer) in order to fix and remove the brain. Once the animal was deep under anaesthesia and completely unresponsive to pain, the thoracic cavity was opened to expose the heart. A needle was inserted and secured into the left ventricle, and a heparinised saline solution was perfused. Following the saline perfusion, the fixative (PFA) was perfused and the brain removed. Following perfusion, the brains were removed and 40 μm coronal sections through the NAc (+3.70 to +0.20 AP from Bregma) were prepared using a microtome. The location of the electrode following the final relapse test was assessed through the use of cresyl violet staining.

3.8.2 Cresyl violet staining. The coronal sections of saccharin animals were processed with cresyl violet stain in order to confirm the location of the tip of the electrode. Coronal slices were mounted from 0.1 M PB solution onto gelatine-coated slides and then air dried overnight. The slides were delipidised in varying solutions of ethanol, first with 10 dips in 70% ethanol, 10 dips in 95% ethanol, 10 dips in 100% ethanol, the slides were left in 100% ethanol for 5 minutes, then dipped in

95% ethanol 10 more times and finally left in 70% ethanol for another 5 minutes. The slides were then rehydrated through rinsing for 1 minute in distilled water before incubation in the stain 0.5% Cresyl Violet acetate (250 ml) for 5 minutes. To remove any excess stain, the slides were then rinsed in 2x2 minute dips into distilled water. Each slide was then dehydrated and differentiated in 70% ethanol for 2 minutes, then 95% ethanol for another 2 minutes, this was followed by 40 seconds exposure to 95% acid alcohol (400 ml ethanol with 1 ml glacial acetic acid) and 2x4 minutes exposure to 100% ethanol. Each slide was then cleaned in 2x5 minute exposures to xylene before being cover slipped using DPX.

3.8.3 Verification of electrode placement. Only animals with electrode placement in the area of interest (NAc) were included in any data analysis. Photomicrographs were taken of the NAc regions with evidence of the electrode for each animal with a Nikon camera mounted and studied on a light microscope (Zeiss Axiostar Plus, Germany).

4.0 Results

4.1 Data acquisition

During self-administration tasks, data was recorded for the number of active and inactive lever presses, and the total number of reinforcements based on the ratio of responses to reward (i.e., FR1, FR2, or FR3). This information was gathered for each self-administration session the animals took part in and stored in Excel spreadsheets.

4.2 Statistical analysis

Using the statistical programme StatView 5.0, the data was evaluated through analyses of variance (ANOVA's). Repeated measures ANOVA was used to determine significance between training and testing phases, with drug treatment (cocaine or saline) and stimulation parameters (HF, LF and sham stimulation) as between-groups factors. Tukey-Kramers' post-hoc comparisons were used to examine the significant differences obtained from the ANOVA between the drug conditions.

4.3 Self-administration training

Figure 5-A shows the number of active and inactive lever presses for all groups in the five day training phase for self-administration. Figure 5-B displays the average number of reinforcements obtained by each group during the training phase. Compared to saline control animals, all of the experimental cocaine animals show significantly higher responding in the active lever ($F(3, 26) = 20.238$, $p < .0001$) with no difference in inactive lever responses ($F(3,26) = 0.276$, $p = .824$). It is clear that the animals were able to discriminate cocaine from no infusion, as there was a greater number of responses on the active lever.

4.4 Intake test

Following training, all animals took part in an intake test, comprising of 30 min of the corresponding stimulation followed by a 90 min intake test, where cocaine or saline was available following response on the active lever on a FR3 schedule. Figure 5-C displays the number of reinforcements obtained during the intake test for all groups. Tukey-Kramers' post-hoc test revealed that there were no significant differences between the experimental groups, with a significant increase only found between the SA-Sham and the three cocaine groups ($F(3,26) = 24.245$, $p < .0001$). These data showed that DBS exposure prior to cocaine self-administration had no significant effects on cocaine intake.

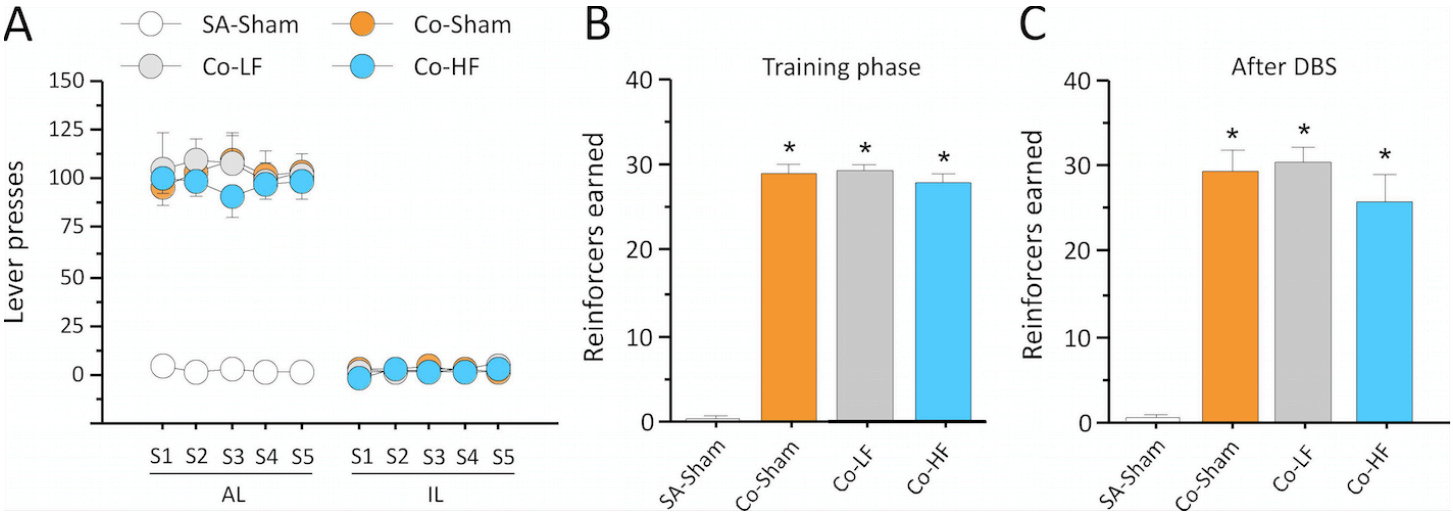

Figure 5. A) Active and inactive lever presses for the five stable days responding for cocaine or saline self-administration. B) Average number of reinforcements obtained over the training phase of self-administration. C) Number of reinforcements obtained during the intake test following a 30 min session of stimulation. Error bars show SEM. SA-Sham = saline-sham stimulation; Co-Sham = cocaine-sham stimulation; Co-LF = cocaine-low frequency stimulation; Co-HF = cocaine-high frequency stimulation; * = significantly different ($p < .05$) from saline.

4.5 Relapse tests

We found an interaction effect in the ANOVA, between the relapse tests (days 1, 15 and 30), lever presses (active, inactive) and the groups of the animals (SA-Sham, Co-Sham, Co-LF, and Co-HF) ($F(2,52) = 5.657$, $p = .006$).

Figure 6-A. shows the differences revealed by Tukey-Kramers' post-hoc analysis. These differences were significant when comparisons were made between the Co-Sham and Co-LF experimental groups ($p < .05$) as well as between the Co-Sham and Co-HF experimental groups ($p<.05$) on day 15 of the withdrawal period. These results shows that DBS treatment had a significant effect on the number of responses to the cocaine associated lever following 15 days of withdrawal and stimulation.

4.6 Food motivation training and intake tests

There were no differences between the groups in number of reinforcers earned following five stable days of saccharin self-administration training ($F(1,10) = .596$ $p = .458$). The animals were split into two counterbalanced groups, which took part in two separate intake tests (all animals took part in both LF and HF sessions followed by an intake test at least 48 h apart with training sessions in between to return to stable responding for saccharin), Figure 6-B. shows the reinforcements obtained for both stimulation sessions as well as the baseline training phase. There was no difference found between the intake of saccharin following either HF or LF stimulation sessions ($F(1,10) = 0.596$, $p = .458$). These observations indicate that DBS therapy had no significant effect on the responding for saccharin reinforcers.

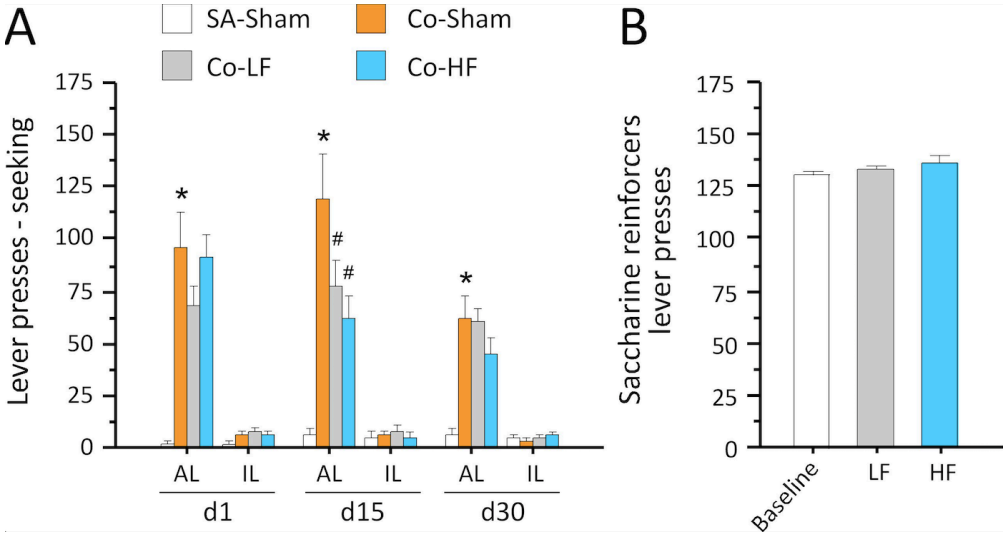

Figure 6. A) Active and inactive lever presses for the relapse sessions (day 1, 15, and 30) for cocaine and saline animals. B) Saccharin reinforcements during baseline training and intake tests following 30 min LF and HF stimulation. Error bars show SEM. SA-Sham = saline-sham stimulation; Co-Sham = cocaine-sham stimulation; Co-LF = cocaine-low frequency stimulation; Co-HF = cocaine-high frequency stimulation; AL = active lever; IL = inactive lever; * = significantly different ($p < .05$) from saline; # = significantly different ($p < .05$) from CO-Sham on day 15.

4.7 Histological determination of electrode placement

Figure 7-A shows a representative photomicrograph of a section through the nucleus accumbens stained with cresyl violet. Based on the photomicrographs, four animals were discarded due to incorrect placement of the electrode (i.e., in all four cases the electrode protruded beyond the ventral aspect of the NAc), therefore any stimulation through the tip of the electrode would have missed the intended target. Figure 7-B shows the representative placement of each electrode in the nucleus accumbens, including all included and excluded animals.

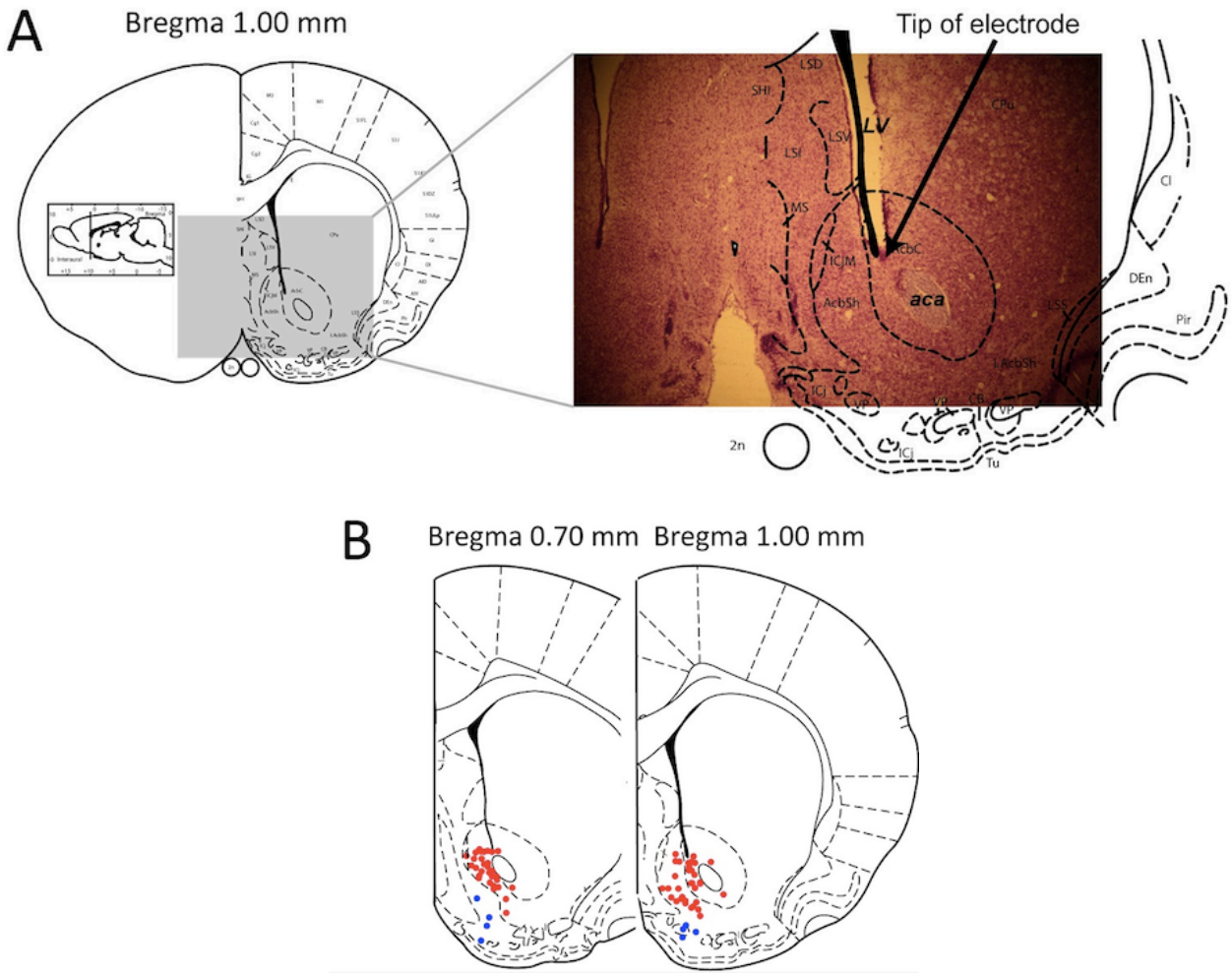

Figure 7. A) Photomicrograph of cresyl violet stained coronal section showing the electrode placement within the nucleus accumbens in one experimental animal. B) Electrode placement for each animal. Red dots = all animals included in data analysis. Blue dots = animals excluded from the experiment and analysis due to electrode placement.

5.0 Discussion

5.1 Discussion

The aim of the current experiment was to examine the beneficial effects of chronic exposure to unilateral LF and HF stimulation of the NAc on cocaine relapse. Animals pre-trained on cocaine self-administration of a FR3 schedule of reinforcement were treated with either LF (20 Hz), HF (160 Hz) or sham (0 Hz) stimulation during a forced-abstinence period of withdrawal and then tested for cocaine seeking. To model a chronic treatment situation, each group experienced 15 consecutive days of DBS treatment (30 min: sham, LF or HF). First, the results showed that there were no immediate effects of DBS on the intake of cocaine, with all animals responding similarly in the intake test conducted after a 30 min session of sham, LF or HF stimulation. Second, the self-administration context paired with cocaine challenge induced robust cocaine seeking in three relapse tests performed on days 1, 15 and 30 after withdrawal from cocaine self-administration. Relapse rates were greatest 15 days after withdrawal. Tests conducted on day 1 and 30 revealed no significant difference between the sham stimulated and LF and HF stimulated groups. However, on day 15 of withdrawal, chronic exposure to DBS produced a significant attenuation of cocaine seeking behaviour in both the LF and HF stimulation groups, with the HF stimulation parameters being apparently more effective. The results support the hypothesis that chronic exposure to unilateral LF and HF stimulation attenuates cocaine relapse following a period of forced-abstinence.

The first relapse test took place after a single exposure to stimulation; therefore we were able to determine that the beneficial effects of both LF and HF DBS are not apparent following one session of stimulation, with inability to attenuate cocaine seeking in cocaine-experienced animals. DBS effectively attenuated relapse after 15 days of repeated stimulation, but it appears as though the effects diminished over the second phase of the withdrawal period, that is,15 days following the cessation of stimulation, with no effects being apparent in the LF or HF stimulated groups in the final relapse test (day 30). Hu and colleagues (2011) demonstrated the reversal of the beneficial

DBS effects with evidence that any effects ceased almost immediately following cessation of stimulation. We can conclude that any effects, negative or beneficial following DBS treatment, appear to be temporary. Future work should be aimed at collecting relevant electrophysiological data from brain areas within the reward system to fully understand the nature of the functional changes before, during, and after DBS. Adding these observations onto the behavioural information collected would help in the determination of exact mechanisms at work and the most beneficial timeline for significant reduction of cocaine craving.

Ewing and Grace (2013) established that long-term exposure to DBS has altering effects on the systems within and extending from the NAc. Both LF and HF stimulation exposure for 15 days was sufficient to significantly attenuate cocaine-seeking behaviours in the current experiment, implying an both manipulations were able to produce disruptions of the functional networks subserving cocaine-motivated behaviours. Moreover we were able to demonstrate positive effects on behaviour on day 15 following chronic administration of DBS, however the effects diminished following a 15 day period of withdrawal from DBS treatment. This suggests the need for stimulation therapy to be applied for an extended period of time with daily, uninterrupted exposure. Further elaborations of this experiment could examine the time-course to determine ideal administration and continuation of DBS treatment to reduce cocaine craving.

Unilateral stimulation in animals undergoing relapse to drug seeking constitutes a relatively novel approach to the treatment of drug craving and addiction. The results of this study provide evidence of a significant effect seen with unilateral application of both LF and HF stimulation to the NAc. Alberts and colleagues (2008) have demonstrated the feasibility of this approach with evidence of patients reducing medication for the neuropsychiatric disorder PD, following unilateral stimulation, and although preliminarily, conclude that more effort needs to be granted to the study of unilateral implantation of electrodes. A replication of the current study with bilateral

implantation of stimulating electrodes would elude the difference in beneficial effects between unilateral and bilateral stimulation using the same addiction and treatment paradigms.

Vassoler and colleagues (2008, 2013) have provided evidence that HF stimulation alters cocaine-seeking behaviours when applied to the shell of the NAc, an effect that is likely to be mediated through both antidromic and local activation. Through comparisons of DBS with pharmacological inactivation of the NAc shell, these authors were able to dissociate the effects of the differing forms of activation (i.e., antidromic vs. local). They suggested that there is incomplete activation of the GABAergic interneurons through cortico-accumbal afferents. There is evidence of DBS activating axons innervating the stimulation regions (McIntyre *et al.,* 2004) with DBS of the NAc suppressing neuronal activity in the orbitofrontal cortex via antidromic stimulation of cortico-accumbal afferents and the subsequent activation of the inhibitory cortical interneurons (McCracken & Grace, 2007). Although the effects of LF stimulation are not as well understood that those induced by HF, Hu and colleagues (2011) demonstrated that a range of frequencies attenuated neuronal firing in the region of the NAc, although they showed that frequencies on the lower end of the scale (lower than 50 Hz) produced less suppressive effects than higher frequencies (e.g., 130 Hz). In our experiments, the HF stimulated group exhibited greater attenuation of cocaine seeking following chronic exposure than LF, which may be indicative of increased suppressive effects of HF stimulation on neuronal discharge frequency.

There is limited research on the use of unilateral LF and HF stimulation of the NAc for use in addiction, as the majority of studies published so far have investigated the effects of bilateral, HF stimulation (Vassoler *et al.,* 2008, 2013; McCracken & Grace, 2007). It is of great importance that significant attenuation of cocaine seeking behaviours was found in stimulated animals (LF and HF) compared to a sham stimulation control group. The demonstration of similar results between LF and HF stimulation groups on all test days indicates that both stimulation parameters (and potentially

others in between) should be investigated to determine an optimal stimulation treatment for addiction patients.

When manipulations are introduced to reduce or block certain behaviours, such as drug self-administration or drug seeking, it is important to ensure that these behaviours are affected specifically. In this study, we examined the effects of LF and HF stimulation on the self-administration of saccharin to control for the possibility that DBS alters motor and/or motivational functions in general. Our observation that there was no change between the number of saccharin reinforcers administered in self-administration training and the intake tests indicated that neither LF nor HF stimulation had undesirable effects on motivation and motor performance.

Due to the effects of stimulation only being apparent at day 15 after withdrawal from cocaine self-administration, we were unable to examine changes in gene and protein expression at that specific time point, as the brains were not removed until day 30. Without such potentially relevant neurochemical and physiological evidence, we can only speculate, based on previous research, on the potential circuits and structures involved in the attenuation of cocaine-seeking behaviours. A replication of these experiments incorporating measures of gene/protein expression and electrophysiological recordings would provide a great deal of insight into the mechanism underlying the beneficial effects associated with DBS of the NAc. By using a between-subjects design with each animal taking part in one relapse test rather than all three (either day 1, 15 or 30), more definitive and reliable behavioural results may be obtained due to the absence of carry-over effects. This would also allow for neurochemical analysis to be performed after each relapse test.

Furthermore, it is important to determine the effects of both LF and HF stimulation of different areas in the NAc to identify the most suitable target regions for therapeutic stimulation to be applied. Previous studies have differentiated the two areas within the NAc (Vassoler *et al.,* 2008, 2013), however these studies only applied HF stimulation parameters to reduce drug-seeking behaviours after chronic self-administration.

5.2 Future directions in deep brain stimulation therapy

Given the current results with DBS together with the previous data from other laboratories, it appears that both LF and HF stimulation provides a promising therapeutic avenue for patients with stimulant addiction disorders. There is a great deal of evidence that DBS therapy has benefits for patients with treatment resistant disorders such as PD, OCD and movement refractory disorders (Wichmann & Delong, 2006), with preliminary evidence in addiction disorders showing potential.

New animal research currently under way is aiming to determine the exact mechanism involved in DBS in order to fully utilise its therapeutic potential. Vassoler and colleagues (2013) have recently unveiled the putative mechanisms of HF stimulation within the NAc shell, suggesting that both local and antidromic activation is involved in the attenuation of cocaine-seeking behaviours. Electrophysiological evidence shows that NAc DBS inhibits glutamatergic cortico-accumbal activity while also stimulating cortical interneurons. While these data further expands our knowledge surrounding DBS, it also highlights the complexity of the reward systems involved.

Extensive cellular recording is currently being utilised to generate a real time mapping of the changes occurring during sessions of stimulation. Local field potentials in regions including the NAc can help determine the exact changes in the signals during and following stimulation therapy. Previous work in electrophysiology has indicated that the physiological changes induced by a single HF stimulation session reverse almost immediately (Hu *et al.,* 2011), which would suggest little clinical application unless exposure is 24 hours a day. Ewing and Grace (2013) discuss the importance of long-term exposure to stimulation in order to generate the greatest benefits for the individual.

Clinical experiments are also under way in order to validate DBS as a safe and effective form of treatment in human addiction patients. Clinical trials, analogous to animal studies, have focussed on the NAc as the most effective target for DBS in order to reduce addictive type

behaviours (Mantione *et al.,* 2010; Kuhn *et al.,* 2011; Zhou *et al.,* 2011). Animal research into DBS therapies has great utility for identifying mechanisms and targets for effective stimulation in neuropsychiatric disorders such as psychostimulant addiction. It is important to study the effects of DBS therapy in human addicts to add to the growing data from animal models to ensure that therapeutic effects seen in animals are translatable into the human population.

5.3 Conclusions

Cocaine is a highly addictive psychostimulant with great abuse potential and associated with the high levels of euphoria compelling an individual to repeat administration. Due to this strong addictive property and the lack of efficacious pharmacological agents available for treatment, there is a great demand for potential therapies in stimulant drug addiction. Indeed, current pharmacotherapies and cognitive therapies are not effective for many addicted individuals, which calls for the development of stimulation therapies. DBS has recently emerged as a potential therapy for addiction disorders, with a great deal of efficacy apparent in other disorders including PD and OCD. The current research has built on previous work in animal models of cocaine addiction, observing the effects of chronic exposure to unilateral LF and HF stimulation on relapse to cocaine seeking. This study determined an attenuation of relapse to cocaine on day 15 of withdrawal but not days 1 or 30. The findings support the claim that extended periods of stimulation could generate physiological changes in the systems within and extending from the NAc, with the effects diminishing following the cessation of stimulation therapy. Further work into chronic exposure to DBS with additional electrophysiological data to complement the behavioural changes will aid in the delineation of the circuitry involved in the attenuation of cocaine craving. Both animal and clinical research into DBS treatments for addictive disorders is developing at a fast pace, bringing new hope for treatment refractory individuals.

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7.0 Appendices

7.1 Appendix A. Ethics Approval

UNIVERSITY OF CANTERBURY
Te Whare Wānanga o Waitaha
CHRISTCHURCH NEW ZEALAND

ANIMAL ETHICS COMMITTEE

Secretary, Lynda Griffioen
Email: animal-ethics@canterbury.ac.nz

Ref: 2012/35R

17 December 2012

Jungah Lee & Jennifer Hamilton
Department of Psychology
UNIVERSITY OF CANTERBURY

Dear Jungah and Jennifer

I am pleased to inform you that the Animal Ethics Committee (AEC) has approved your application entitled: "Deep brain stimulation of the nucleus accumbens for the treatement of drug addiction".

Approval has been granted:
(a) for the use of 66 Long Evans Rats - Male
(b) for your research project to be undertaken from 1 February 2013 to 31 January 2014. If you require an extension of this period please contact the AEC Secretary.
(c) This approval is subject to providing the AEC with a progress report following the first experiment.

As part of AEC's new Code of Ethical Conduct all applicants receiving approval to work on animals are required to provide a final report at the completion of their project. The purpose is to provide the AEC with a record of your use of animals and what was achieved by your research project. We are very much interested in your findings and to learn what you have achieved. Following the completion date indicated above you are asked to provide this report using the new Final Report form which is available at the AEC web site (https://intranet.canterbury.ac.nz/research/ethics.shtml).

On an annual basis the University is legally required to provide to MAF statistical data on all animal manipulations undertaken in a calendar year. To assist us in collating this information you are also required to complete and return to the AEC Secretary the attached MAF Animal Manipulation Statistical form 30 days after the completion of this project, or once every three years, which ever comes first. If no animals have been manipulated in your project please provide a "Nil" return. Please also find enclosed a copy of the Animal Welfare (Records and Statistics) Regulations 1999 for your information, together with a list of Animal Type Codes and brief guideline notes for your assistance.

Yours sincerely

Associate Professor Jim Briskie
Chair
University of Canterbury Animal Ethics Committee